Supplemental Information

Supplemental Figure Legends

Figure S1: Normal morphology, survival, and synapse formation in neurons expressing Bassoon/Piccolo DKD shRNAs. A) Cell bodies and dendrites from 14 DIV neurons infected with scrambled control (EGFP/SC) or Bsn16/Pclo28 (EGFP/DKD)(green) at 0 DIV and immunostained with antibodies to MAP2 (red). Size bar is 25 μm. B) Quantification of primary dendrite number in uninfected cells and those infected with EGFP/SC or /DKD. No significant difference was observed between the 3 conditions (n=22 cells/condition; t-test). C) Dendrites from 19 DIV neurons infected with EGFP/SC or EGFP/DKD at 0 DIV. Note that dendritic spine morphology is similar for both conditions. Size bar is 10 μm. D) Quantification of spine density in 19 DIV neurons infected with EGFP/SC or /DKD, expressed as spines per pixel length of dendrite. No significant difference was seen between the two conditions (n=12 fields of view/condition, with ~3-4 dendrites/field; t-test). E) Images of EGFP, EGFP/SC or /DKD infected neurons from the same fields of view, imaged weekly for 2 weeks to track cell survival. Red asterisks denote neurons that died between 7 and 14 DIV. Size bar is 200 μm. F) Quantification of cell survival at 14 DIV, expressed as fraction of surviving cells. There is no significant difference in cell survival between the EGFP alone, EGFP/SC, or EGFP/DKD condition (n= 4 fields of view/condition, representing 100-200 total cells/condition; t-test). G) Images of 14 DIV neurons expressing EGFP-SV2 together with scrambled shRNAs (SC) or Bsn16/Pclo28 (DKD), immunostained with PSD-95 antibodies to label postsynaptic densities. Arrows indicate a subset of the EGFP-SV2 puncta that colocalize with PSD-95. Note that a majority of EGFP-SV2 puncta colocalize

with PSD-95 in both SC and DKD conditions. Size bar is 10 μm. H) Quantification of EGFP-SV2 colocalization with PSD-95 for SC and DKD conditions. No significant difference in the fraction of colocalization was observed (n>6 fields of view/condition, >100 puncta/field, unpaired t-test).

Figure S2: Pre- and postsynaptic markers are reduced in DKD boutons. A) Images of 14 DIV neurons expressing EGFP-Synapsin1a (EGFP-Syn) together with SC or DKD shRNAs, immunostained with synaptophysin antibodies. Note that the size and fluorescence intensity of EGFP-Syn puncta, like EGFP-SV2 puncta, are significantly reduced at DKD boutons. Size bar is 10 μm. B) Quantification of EGFP-Syn colocalization with synaptophysin for SC and DKD conditions. Significantly fewer EGFP-Syn puncta colocalize with synaptophysin in the DKD (~30% vs. 70%; n> 3 fields of view/condition, with >50 puncta/field; * = $p<0.05$, unpaired t-test. C) Images of 14 DIV neurons expressing EGFP-Syn/SC and /DKD, loaded with FM4-64 using 10Hz stimulation for 60 sec. Size bar is 10 μm. D) Quantification of EGFP-Syn colocalization with FM4-64 for SC and DKD conditions. Significantly fewer EGFP-Syn puncta colocalize with FM4-64 in DKD boutons (~80% vs. ~40%; n>5 fields of view/condition, >50 puncta/field; ***= $p<0.0001$, unpaired t-test. E) Quantification of fluorescence intensities for a series of synaptic markers at EGFP-Syn/DKD boutons, expressed as fractions of fluorescence intensity for the same marker at EGFP-Syn/SC boutons. Dotted line at 1.0 indicates the average fractional intensity for all markers at SC control boutons (ie. SC/SC). Note that presynaptic markers (SV2, synaptophysin, FM4-64) are reduced by ~50% at DKD boutons, while postsynaptic PSD-95 is reduced by ~25% and Homer1b

(postsynaptic) remains unchanged (n>4 fields of view/condition, >30 boutons/field; ** = p<0.005, ***= p<0.0001, unpaired t-test). F) Cumulative frequency distribution of SV2 immunofluorescence intensity at EGFP-Syn/SC (black line) or /DKD (green line) expressing boutons. Note that the distribution is shifted to the left for DKD boutons, indicating smaller SV pool size. SV2 intensity values are from E (n=332 DKD and 360 SC boutons from 5 fields of view). G) Similar to F, but for synaptophysin (n=635 DKD and 369 SC boutons, 7 fields of view). H) Similar to F, but for FM4-64 (n=254 DKD and 177 SC boutons, 8 fields of view). I) Cumulative frequency distribution of PSD-95 immunofluorescence intensity at postsynaptic sites juxtaposed to EGFP-Syn-expressing boutons. The distribution of PSD-95 intensity is also slightly left-shifted for DKD boutons. Values are also from E (n=144 DKD and 251 SC boutons, 4 fields of view. J) Similar to I, but for Homer1b. Interestingly, Homer1b puncta intensity distribution is nearly identical for SC and DKD-expressing boutons (n=119 DKD and 221 SC boutons, 4 fields of view).

Figure S3: Presynaptic proteins have faster degradation rates in DKD neurons. A) Immunoblots of EGFP/SC or EGFP/DKD-expressing neuronal lysates, collected from 13 DIV neurons treated for 0, 12, or 24 hours with cycloheximide, and probed with the antibodies indicated. B-D) Intensities of VAMP2, SNAP-25, and Munc13 immunoreactivity, respectively, at each timepoint (normalized to tubulin and expressed as a fraction of initial intensity at time 0). Note that all three proteins have significantly decreased intensity in DKD vs. SC neurons after 24 hours of cycloheximide treatment (*=p<0.01, t-test; 3 experiments).

Figure S4: Polyubiquitination mediates the degradation of SV-associated proteins. A) 14 DIV neurons singly transduced with EGFP-SV2/SC or /DKD at the time of plating, or co-transduced with EGFP-SV2/SC or /DKD and knockout (KO) ubiquitin expressed together with mCherry (+KO-Ub/mCherry), fixed and immunostained with VAMP2. Arrows indicate a subset of EGFP-SV2 puncta that colocalize with KO-Ub and VAMP2 in presynaptic boutons. VAMP2 fluorescence in both the SC and DKD backgrounds is visibly increased by KO-Ub. Size bar is 10 μm. B) Quantification of VAMP2 intensity in SC and DKD boutons +/- KO-Ub. Values are expressed as fraction of VAMP2 intensity at untreated EGFP-SV2/SC boutons (dashed line). KO-Ub increases VAMP2 fluorescence by ~20% at SC boutons and by ≥30% at DKD boutons (n≥4 fields of view/condition, >50 puncta/field; **=$p<0.005$, ***=$p<0.0001$, unpaired t-test). Results were confirmed in 3 independent experiments. C) Same as B, but for synaptophysin intensity. KO-Ub increases synaptophysin fluorescence by ~20% at SC boutons and by ~30% at DKD boutons (n≥4 fields of view/condition, >50 puncta/field; **=$p<0.005$, ***=$p<0.0001$, unpaired t-test). Results were confirmed in 3 independent experiments.

Figure S5: Bassoon and Piccolo zinc fingers attenuate Siah1 activity. A) Schematic diagram depicting the segments of Bassoon used in a yeast two-hybrid screen to identify interacting partners. The N-terminal segment of Bassoon containing both zinc fingers (Zn1, Zn2) (RB31; amino acids 1-609) exhibited strong binding to Siah. All other Bassoon segments, covering regions downstream of reside 609, did not show any binding to the clone containing the full-length cDNA of Siah1. The cDNA clone of rat Siah1 (NM.080905) that was originally isolated as an interacting partner of the RB31 segment

of Bassoon covered a sequence starting at amino acid 59, containing part of the RING domain (which spans amino acids 39-76), and part of the subsequent Sina domain. B) 14 DIV neurons expressing EGFP-SV2/SC or /DKD together with either soluble mCherry or one of the two mCh-tagged Piccolo Zinc fingers. Size bar is 10 μm. C) Quantification of EGFP-SV2 fluorescence intensity in SC or DKD boutons co-expressing soluble mCh or one of the four Bassoon or Piccolo mCh-tagged ZFs. Note that each ZF partially rescues EGFP-SV2 fluorescence (by ~30%) in the DKD background (n=3 fields of view/condition, >60 boutons/field; ***=$p<0.0005$, **=$p<0.005$, *=$p<0.05$, unpaired t-test). D) Cumulative frequency distribution of EGFP-SV2 fluorescence intensity in DKD boutons expressing the ZFs. Each ZF partially rescues the DKD phenotype, shifting the intensity distribution toward the right. The distribution of EGFP-SV2 in SC control boutons expressing mCherry is shown for comparison (black curve). Some of these data are also shown in Figure 8 (n=350 SC/mCh, 215 DKD/mCh, 260 DKD/BsnZF1, 130 DKD/BsnZF2, 130 DKD/PcloZF1, 190 DKD/PcloZF2 boutons). E) Western blots showing synaptophysin (Syph) ubiquitination in the presence or absence of Siah1 and Bassoon ZF1 (BsnZF1). Upper two panels show total lysates probed with either myc antibody to detect Siah1, or mCh antibody to detect BsnZF1. Unrelated lane is blocked out with gray background. Lower two panels show immunoprecipitated synaptophysin, probed with HA antibody to detect ubiquitination and mouse synaptophysin antibody to detect total synaptophysin levels. Unrelated lane is blocked out with gray background. Based on the observed size shift of synaptophysin (from 37 to 50 kD), it appears that monoubiquitination of synaptophysin by Siah1 is predominant in HEK cells. F) Quantification of E. HA-ubiquitin intensity is divided by total synaptophysin intensity for

each condition, giving relative HA-ubiquitin levels for synaptophysin. Note that Siah1 increases synaptophysin ubiquitination above baseline, and that BsnZF1 blocks this effect (n=3 experiments, *= $p<0.05$, unpaired t-test).

Figure S6: Characterization of Siah1 shRNA knockdown and EGFP-Siah1 overexpression. A) Western blot of lysates from HEK293 cells transfected with EGFP-Siah1 +/- sh-Siah1, probed with EGFP and neomycin antibodies (to control for vector level). Note that EGFP-Siah1 expression is dramatically reduced in the presence of sh-Siah1. B) Quantification of A. Intensity of EGFP-Siah1 is normalized to neomycin intensity and expressed as a fraction of control (EGFP-Siah1 only). EGFP-Siah1 is reduced almost 90% by sh-Siah1. This experiment was performed three times. C) Images of axons expressing EGFP-Syn/SC or /sh-Siah1, immunostained with synaptophysin and Bassoon. Siah1 knockdown does not affect EGFP-Syn clustering or its colocalization with syph and Bassoon (arrows), indicating that presynaptic boutons still form normally in the absence of Siah1. Size bar is 10 μm. D) 14 DIV axons infected with soluble EGFP or EGFP-Siah1, immunostained with synaptophysin and synapsin antibodies. Arrows indicate presynaptic boutons in processes expressing EGFP or EGFP-Siah1, while arrowheads indicate boutons in uninfected processes. Note the absence of significant synaptophysin and synapsin immunostaining in the Siah1-expressing axon. Size bar is 10 μm. E) Quantification of synaptophysin (Syph) and synapsin (Syn) immunofluorescence intensity in boutons expressing EGFP (black bars) or Siah1 (purple bars). Values are expressed as fraction of intensity at uninfected boutons. Both presynaptic proteins are significantly decreased (by >30%) in axons overexpressing Siah1 (n ≥4 fields of view,

>100 puncta/field; ***=p<0.0005, **=p<0.005, unpaired t-test). F) Quantification of bouton density (expressed as # boutons/pixel length of axon) in axons infected with EGFP or EGFP-Siah1. The density of presynaptic boutons, defined as sites immunopositive for both synaptophysin and synapsin, is decreased nearly threefold in axons infected with EGFP-Siah1 vs. EGFP alone (n=4 fields of view with ≥80 puncta/field for EGFP; n=6 fields of view, ≥8 puncta/field for EGFP-Siah1).

Supplemental Materials and Methods

Reagents used in main paper. Antibodies against Piccolo (rabbit), Bassoon (mouse), and MAP2 (rabbit and mouse) were used as previously described (Zhai et al., 2000). Tubulin (mouse), PSD-95 (mouse), and HA (mouse) antibodies are from Sigma, synaptophysin (rabbit), myc (mouse and rabbit), and ubiquitin (mouse) antibodies from Santa Cruz, CHMP2b (rabbit) antibody from Abcam, Lysine-48 polyubiquitin (rabbit) antibody from Millipore, GFP (mouse) antibody from Roche, and mCherry (rabbit) antibody from BioVision. SNAP25 (rabbit), VAMP2 (mouse and rabbit), synapsin1 (mouse), RIM1 (rabbit), Munc13 (rabbit), and synaptophysin (mouse) antibodies are from Synaptic Systems. LysoTracker red was purchased from Invitrogen and pRK5-Ubiquitin-KO plasmid from Addgene (plasmid 17603). Unless otherwise indicated, all other chemicals are from Sigma. Drugs used in this study (chloroquine, leupeptin, epoxomicin, ziram) were made up as 1000X stocks in DMSO unless otherwise indicated.

Reagents used in supplemental figures. Antibodies against Homer (rabbit) are from Synaptic Systems, and SV2 (mouse) from Developmental Studies Hybridoma Bank (U of Iowa). FM4-64 was purchased from Molecular Probes/Invitrogen. For yeast two-hybrid

experiments, the Matchmaker Two-Hybrid System 2 (Clontech Laboratories, Inc.) was used with an oligo dt-primed pACT2 rat brain Matchmaker cDNA library (Clontech Laboratories, Inc.)

Design of DNA constructs

Creation of tricistronic lentiviral vector for expression of two shRNAs and a reporter protein. Bsn16 was subcloned into pZOff 2.0 (like pZOff 1.0, but with U6 instead of H1 promoter) at the BglII and HindIII sites. To express both Bsn16 and Pclo28 shRNAs, the FUGW H1+ vector was modified in two steps. First, a 750 nucleotide stuffer sequence containing 5' EcoR1 and BstB1 sites was inserted at the Bsiw1 site. Second, the sequence containing the U6 promoter and Bsn16 shRNA in pZOff 2.0, flanked by EcoR1 and Acc1 sites, was subcloned in at the EcoR1 and BstB1 sites. The resulting FUGW vector drives expression of the Pclo28 shRNA via the H1 promoter and, separated by 750 nucleotides, the Bsn16 shRNA via the U6 promoter, an arrangement that allows for efficient lentivirus production and simultaneous knockdown of both Piccolo and Bassoon. EGFP-Synapsin1a, EGFP-SV2, and VAMP2-HRP were subcloned into these FUGW vectors in place of soluble EGFP.

*Siah1 shRNA and lentiviral vectors***.** shRNAs against Siah1 were designed using target sequences obtained from iRNAi (http://www.mekentosj.com/science/irnai) and the rules developed by Reynolds et al. (Reynolds et al., 2004).

Sh Siah1 oligos. 5'-gatccccctgtcgccccaaacttacattcaagagatgtaagtttggggcgacagtttttggaaa-3' (FW) and 5'-agcttttccaaaaactgtcgccccaaacttacatctcttgaatgtaagtttggggcgacagggg-3' (RV).

Oligos contain BglII and HindIII looped overhangs for subcloning into pZOff. Two of these were subcloned into the pZOff vector and tested in HEK cells, and the most effective (sh-Siah1) subcloned into the FUGW H1 vector (Leal-Ortiz et al., 2008) containing mCherry-tagged Synapsin1a in place of soluble EGFP.

*Myc and EGFP-tagged Siah1 constructs***.** Siah1 was cloned from total rat RNA using the SuperScriptIII RT-PCR kit (Invitrogen) according to manufacturer's instructions, and subcloned into the EcoR1 site of EGFP-C2 vector (Clontech) and a modified C2 vector containing the myc tag in place of EGFP. RING and Sina constructs were created by digesting EGFP-Siah1 with Apa1, subcloning the resulting Sina fragment into EGFP-C1 at the Apa1 site and reannealing the original vector to create EGFP-RING.

Siah1 primers. 5'-gccaccatgagccgccagactgc-3' (FW) and 5'gattgccgtttgaacacatgga-3' (RV).

Zinc finger primers. For Bsn ZF1: 5'aaaaagcttccaccatgagcagcaccctgtgtccaatatgcaag (FW), 5'aaaaggatcctcagggccctctgcatctgacagttca (RV);

Bsn ZF2: 5'aaaaagcttccaccatgccgaaggaaagggctgcctgcccactg (FW),

5'aaaaggatcctcagcagcctcttggtttggcagttcagac (RV);

Pclo ZF1: 5'aaaaagcttccaccatgtcgaaaaccatctgtcctctttgcaacaccactg (FW),

5'aaaaggatccttagagctctttgcatctgacagtttaaacagagcc (RV);

Pclo ZF2: 5'aaaaagcttccaccatgcccaaaccagcctgtcctctctgtagaactg (FW),

5'aaaaggatcctaattgctctctgtgtctggcagtttaaacaaagcc (RV).

ShRNA resistant Bassoon. Fragment 1 was amplified with:

Bsn AccIII FW: 5'ccaccggcgcctccggactcag and Bsn 16 silent mutation RV:

5'ggttgaacccacattggttgcagaccttgttgtggcactgggtacaggtattgaaatttggc

Fragment 2 was amplified with Bsn 16 silent mutation FW:

5'cacatcaacctccagccagccaaatttcaatacctgtacccagtgccacaacaagg

Bsn AflII RV: 5'cctccgcactcttaaggggccgtcc. Fragments were purified and put together for the second PCR using Bsn AccIII FW and Bsn AflII RV. The new fragment was subcloned into the original vector at the AccIII and AflII sites.

Untagged synaptophysin for ubiquitination assay. 5'aaaaaggatccgccaccatggacgtggtgaatc (FW); 5'aaaaactcgagttacatctgattggagaaggagg (RV). FW primer contains a BamHI site, RV primer contains an Xho1 site.

mCherry-1D2A-HA-ubiquitin knockout vector. This vector, containing the 1D2A cleavage site (Torres et al., 2010) between mCherry and knockout (KO) ubiquitin, allows for stoichiometric coexpression of mCherry and KO ubiquitin in neurons.

mCherry-1D2A-HA-ubiquitin knockout vector. HA-tagged KO ubiquitin from the pRK5 plasmid was subcloned into the EGFP-N2 vector (Clontech) at the BamH1/Not1 sites, replacing EGFP. The 1D2A site (Torres et al., 2010) was introduced downstream of mCherry in a separate vector using oligos. The mCherry-1D2A cassette was then inserted into the N2 plasmid at the BamH1/Mfe1 sites. Finally, the entire mCherry-1D2A-HA-KO ubiquitin cassette was removed from the N2 vector by Nhe1/Mfe1 digest, and subcloned into the FUGW vector for lentiviral expression at the Xba1/EcoR1 sites.

Electroporation procedure. Cells were spun down and resuspended in glial-conditioned media (10% FBS, 20 mM glucose in glutamine-free MEM; Invitrogen) at a density of $4x10^6$ cells/500 µl of media, placed in 0.4cm Gene Pulser Cuvettes (BioRad Laboratories) along with 40 µg of plasmid DNA, and electroporated at 0.250 kV and 0.975 (µF*1000)

capacitance on a BioRad electroporator. In a number of experiments, additional recombinant proteins and/or shRNAs (ie. RFP-Bsn*, mCherry-tagged ZFs, mCh-Synapsin1a/sh Siah1) were introduced into cells expressing EGFP-SV2/SC or /DKD. For these, cells were electroporated with EGFP-SV2 constructs on 0 DIV, and subsequently infected between 1-5 DIV with lentivirus containing one of these other constructs.

Imaging and image analysis

Quantification of puncta intensity. For each field of view (i.e. image) examined, intensity values for ~100-500 puncta were measured by placing 8x8 pixel boxes around the puncta using the "Place Areas Over Puncta" function in OpenView. In order to capture equivalent numbers of puncta across images of widely varying puncta intensity, the threshold value for this function was not kept constant, but rather set to a value that captured the brightest ~85% of puncta in any given field of view. Measured fluorescence intensity values, reported in arbitrary units, were pasted into Excel for further processing. First, they were individually subtracted from an average background value (obtained from five 8x8 boxes placed randomly over background regions in each image), and all resulting values averaged to give a single value for a given field of view. Values from ~4-8 fields of view were then averaged to give a final intensity value for a given synaptic marker under a particular condition (ie. EGFP-SV2 intensity for SC or DKD boutons). To obtain the fractional values plotted on the bar graphs, each final intensity value was ratioed to that of the SC control value (which was always set to 1 by dividing the value by itself). Cumulative frequency histograms contain intensity values for all puncta from a given batch of neurons (ie. one experiment). Experiments were typically repeated in 2-4

separate batches of neurons.

FM4-64 labeling. FM4-64 labeling experiments were performed during live imaging sessions as previously described (Leal-Ortiz et al., 2008).

LysoTracker puncta quantification. Image analysis was performed in ImageJ as follows: First, any drift in the xy plane was corrected for using the StackReg ImageJ macro and the maximum projection of each z-stack taken. The soma and promixal dendrites of a neuron in the field of view were then selected as an area of interest (in images in which EGFP and LysoTracker fluorescence had been overlayed) using the ImageJ Polygon Selections tool. LysoTracker puncta within that area of interest were defined in a two-step process. First, a threshold of three times the background fluorescence of the LysoTracker image was set to eliminate background fluorescent and define regions of strong Lysotracker signal. Second, in regions where this thresholding failed to distinguish among individual puncta, such puncta were identified manually using the ImageJ Pencil tool. The individuated puncta in the defined area of interest were then numbered and sized by ImageJ's Analyze Particles function. Data is from 15-20 neurons per condition from 4 different cultures.

Quantification of CHMP2b fluorescence intensity. EGFP and MAP2 fluorescence were used to define the region of interest (*i.e.* cell body or axon) for which CHMP2b fluorescence was measured. For cell bodies, ROI was delineated using the polygon selection tool in ImageJ. For axons, only EGFP positive, MAP2 negative processes were chosen for analysis and the "rounded rectangular" tool was used to select a ROI. Intensity values for ≥10 cells or fields of view/condition, measured in arbitrary units, were subtracted from background (obtained by placing smaller rectangles over 3-5 background

regions in the field of view) and averaged to give a single value for each condition. To obtain the fractional values depicted on the bar graph, final intensity values were ratioed to the average SC control value (always set to equal 1).

Quantification of primary dendritic branching and puncta colocalization. Primary dendrites were counted using the ShollAnalysis function in ImageJ, based on EGFP fluorescence and MAP2 immunostaining. Colocalization of EGFP-Synapsin1a with PSD-95, synaptophysin, or FM4-64 was determined using the colocalization macro in ImageJ. Threshold values for each channel were set based on selection of ~80% of the puncta in a given field of view. This method typically underestimates the degree of colocalization by ~10-15%.

Quantification of spine and puncta density. Dendritic spine number or number of synapsin, synaptophysin, or PSD-95 puncta for a given neuronal process were counted manually. The freehand line tool in ImageJ was used to measure the length of each dendrite or axon along which puncta were counted. Number of spines or PSD-95 puncta/unit length dendrite, or synapsin and synaptophysin puncta/unit length axon were calculated in Excel, and resulting values pasted into GraphPad Prism for statistical analysis and graphing.

Visualization/quantification of cell survival. Hippocampal neurons were plated in six-well dishes coated with 500 mg/mL poly-L-lysine and lentivirally transduced with EGFP only, EGFP/SC, or EGFP/DKD at the time of plating. Prior to imaging on DIV7-8, a diamond pencil was used to create reference points on the bottom of the dish, thereby defining fields of view that could be repeatedly identified under bright-field illumination. Four such fields defined in this way were then imaged for each condition and individual

EGFP-positive neurons identified. The same four fields of view were then imaged on DIV14-15, and individual neurons scored at DIV7-8 were identified as being either present or absent at this time point. Data is from 100-200 neurons for each condition from at least three separate cultures.

Quantification of SV number, SV diameter, and PSD number by electron microscopy. HRP-labeled SVs and pleiomorphic vesicles/bouton were counted manually by an experimenter blind to condition (i.e. SC vs DKD). Pleiomorphic vesicles were defined as those with a diameter >80 nm, and SVs as those with a diameter between 30-80 nm. Synaptic vesicle diameter for vesicle clusters within varicosities (presumed to be presynaptic boutons) of 9 DIV neurons was measured in two perpendicular directions using ImageJ. Both clear and HRP-labeled vesicles were included in this analysis, as no significant difference in diameter was observed between the two. Postsynaptic densities (PSDs) juxtaposed to HRP-labeled, vesicle-containing varicosities (presumed to be presynaptic boutons) in the SC or DKD condition were counted manually. For this analysis, 9 and 14 DIV timepoints were pooled.

Biochemistry

Ubiquitination assay: 5 ug of each of the following vectors was co-transfected into 10 cm plates of 80% confluent HEK cells: HA-Ubiquitin, untagged rat synatophysin, myc-Siah1 (or FUGW backbone vector lacking EGFP for control without Siah1), and mCherry-tagged Bsn-ZF1 (or mCh-Synapsin1a for controls without ZF1-mCh). DNA was transfected using 30 ul of CalFectin reagent as described above. 20 hours after transfection, 0.1 uM epoxomicin, 200 uM chloroquine, and 100 uM leupeptin were added

to the cells for an additional 4 hours. Cells were then harvested in 1% Triton X-100 in PBS with protease inhibitor cocktail (Roche) and spun for 30 min at 16,000g to eliminate insoluble material. Immunoprecipitation of synaptophysin was performed using polyclonal antibodies against synaptophysin (Santa Cruz, sc-9116) conjugated to protein A/G beads (Pierce/Thermo Scientific). Following 4 washes in 1% Triton/PBS, bound material was eluted from beads in SDS-PAGE sample buffer (BioRad), separated by electrophoresis, and transferred to PVDF membrane. Resulting Western blots were probed with mouse anti-HA antibody (Sigma) to detect ubiquitination, and with mouse anti-synaptophysin antibody (Synaptic Systems) to detect total synaptophysin levels/sample. The "Gels" application in ImageJ was used to measure intensity of HA and synaptophysin immunoreactivity in each lane. Ubiquitination levels of synaptophysin for each condition were expressed as HA intensity (arbitrary units)/synaptophysin intensity (arb units).

Synaptosome preparation. Crude synaptosomes were prepared from flasks of 9 DIV hippocampal neurons lentivirally transduced with EGFP/SC and EGFP/DKD at the time of plating. The resulting P2 pellet was resuspended in Lysis buffer (50mM Tris-HCl pH 7.2, 150mM NaCl, 1% Triton, 0.5% Deoxycholate, 50uM PR-619 to inhibit deubiquitination, 1 mini cOmplete protease inhibitor (Roche) per 10ml of solution; modified from (Lin et al., 2011)).

Co-immunoprecipitation assay. 10 cm plates of 80% confluent HEK cells were transfected with 5 ug of each vector (ie. mCh-ZF1 and myc-Siah1) using 30 ul Calfectin reagent (SignaGen laboratories) in 1 ml DMEM/condition. Co-IPs with myc-Siah1 and ZFs were performed using a myc immunoprecipitation kit (Pierce/Thermo Scientific).

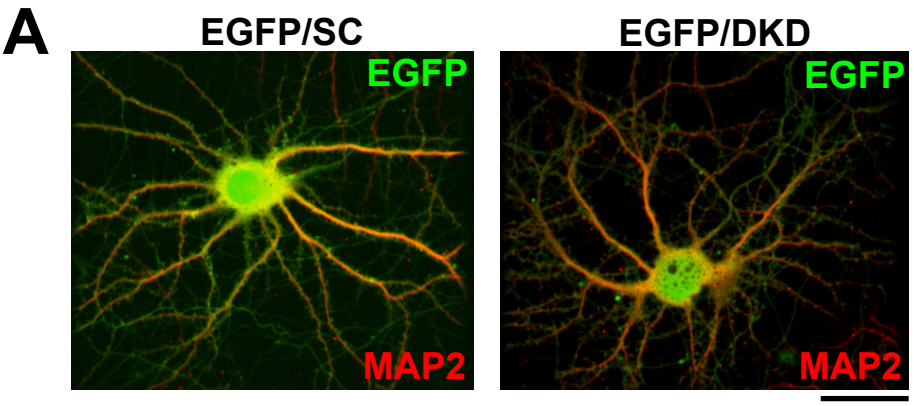
0.5% Tween-20 and 30 μM $ZnCl_2$ in TBS was used at 1:1 with the provided M-PERS lysis solution during 2 hr incubation with myc slurry. Bound material was eluted into the provided sample buffer and immunoblotted as described. Co-IPs with EGFP-RING/Sina and mCh-ZFs were performed in Lysis buffer using the GFP antibody conjugated to DynaBeads. Bound material was eluted from beads into sample buffer, and processed as described above.

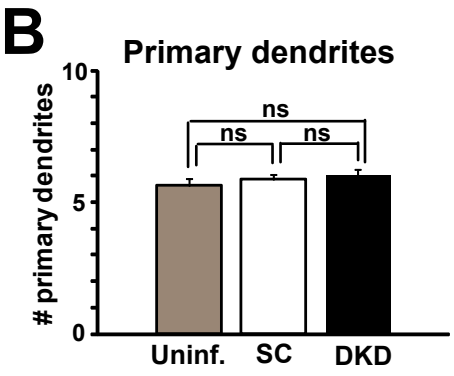
Cycloheximide pulse-chase. 12 DIV neurons infected at 0 DIV with EGFP/SC or EGFP/DKD were treated with cycloheximide (20 μg/ml) and collected at 0, 12, and 24 hours after treatment in sample buffer. Lysates were run on SDS-PAGE and immunoblotted with antibodies against VAMP2, Munc13, and SNAP-25 as described.

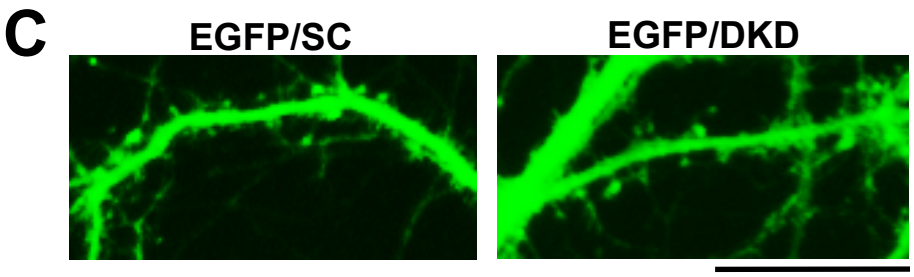
Yeast two-hybrid

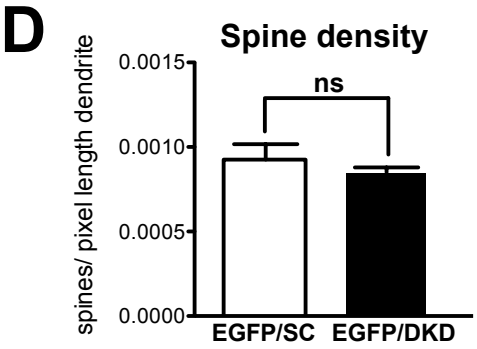
For cDNA library screening, the Matchmaker Two-Hybrid System 2 (Clontech Laboratories, Inc.) was used with an oligodt-primed pACT2 rat brain Matchmaker cDNA library (Clontech Laboratories, Inc.) as prey, and a Bassoon fragment (covering amino acids 1-909 of rat Bassoon) as bait. Transformation and selection was performed according to the manufacturer's instructions. For testing of binding between Siah1 and Bassoon regions downstream of amino acid 609, the Matchmaker system 3 (Clontech Laboratories, Inc.) was used. Specifically, the Bassoon construct (in pGBKT7) was co-transformed with full cDNA of Siah1A in the pGADT7 vector into AH109 yeast cells using standard transformation protocols. Co-transformed cells were selected by growth on Leu- and Trp-lacking medium. The interaction of co-expressed proteins activating expression of reporter genes was monitored as growth on selection media lacking

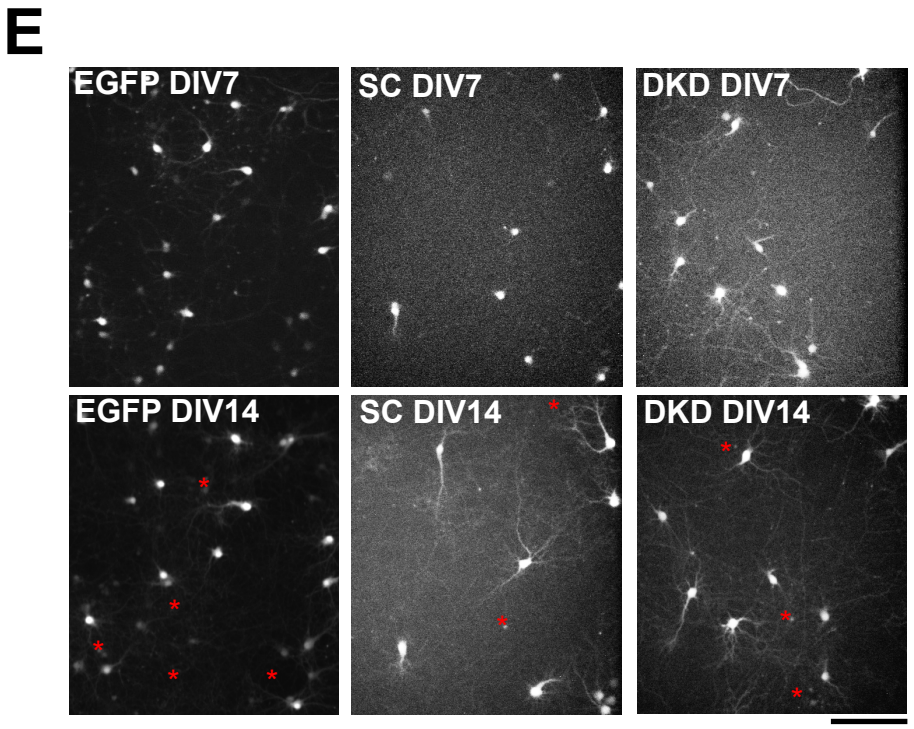
adenine, His, Leu, and Trp and supplemented with 1 mM 3-amino-1,2,4-triazole after 4 and 7 days. Potential self-activation of constructs was always tested in parallel by co-transformation with empty prey or bait vectors.

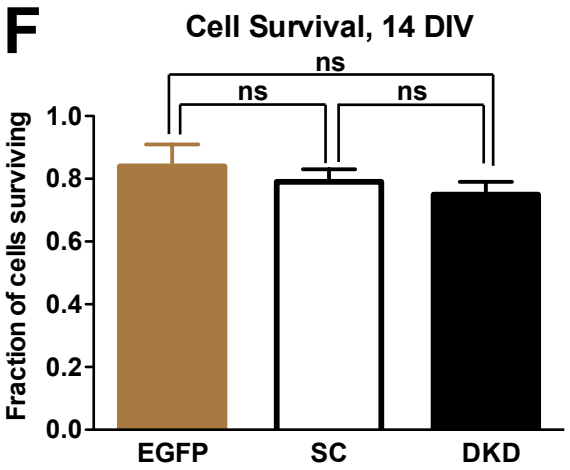


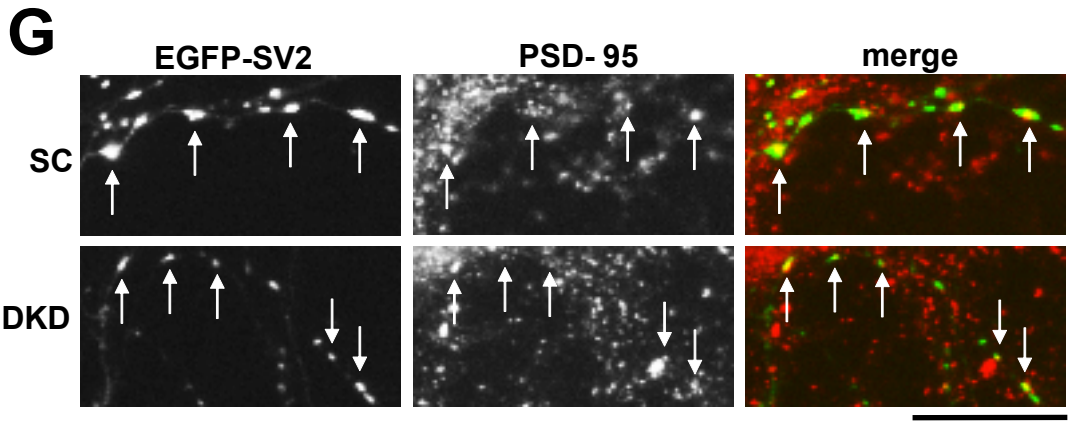


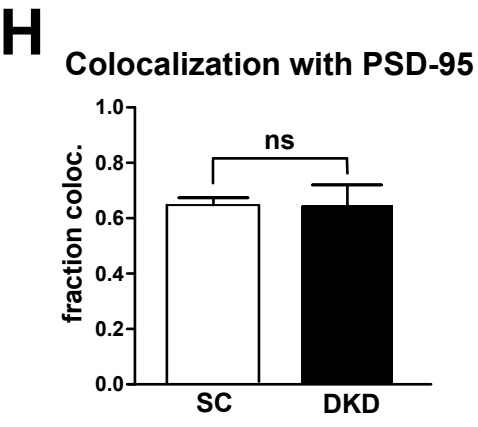




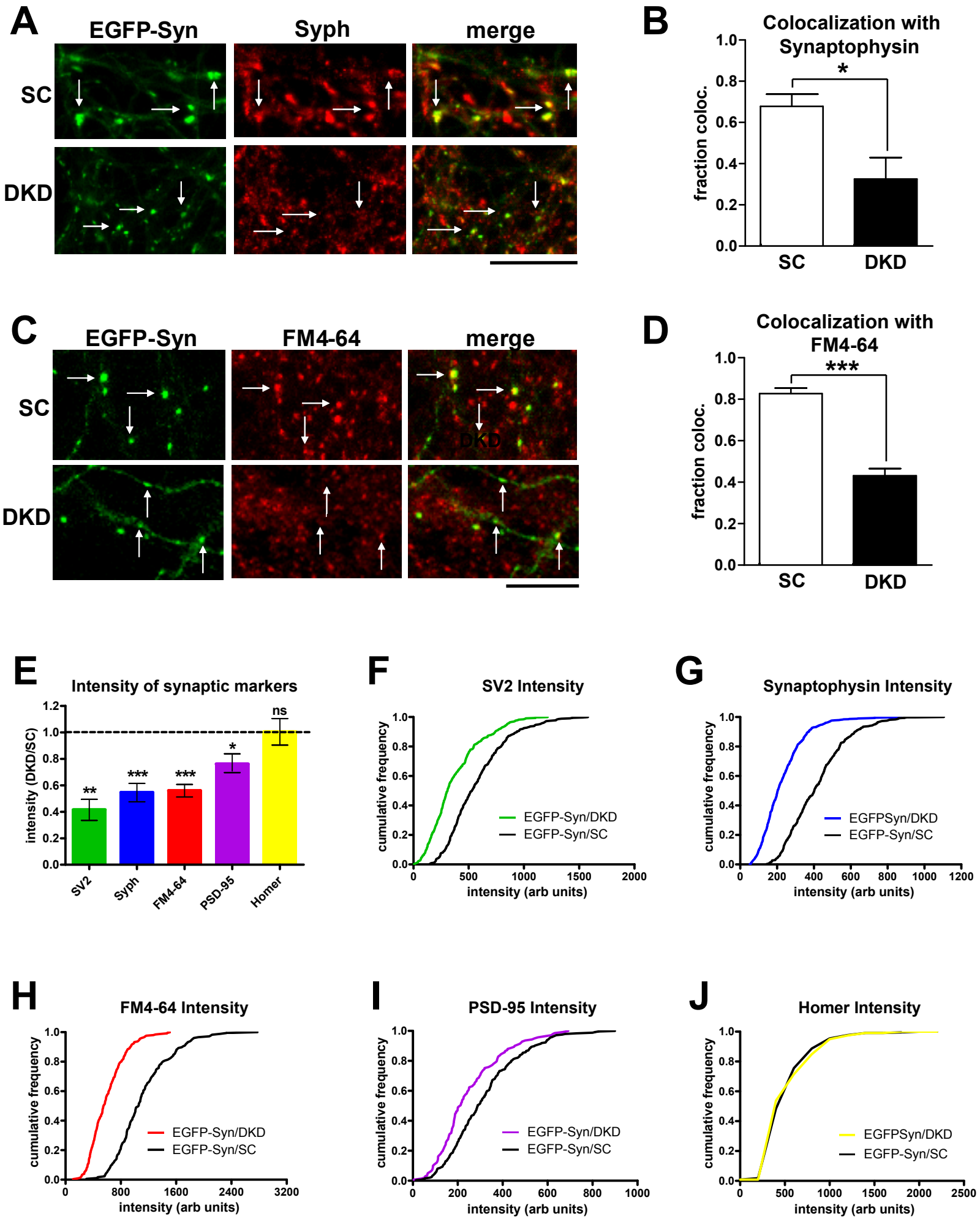




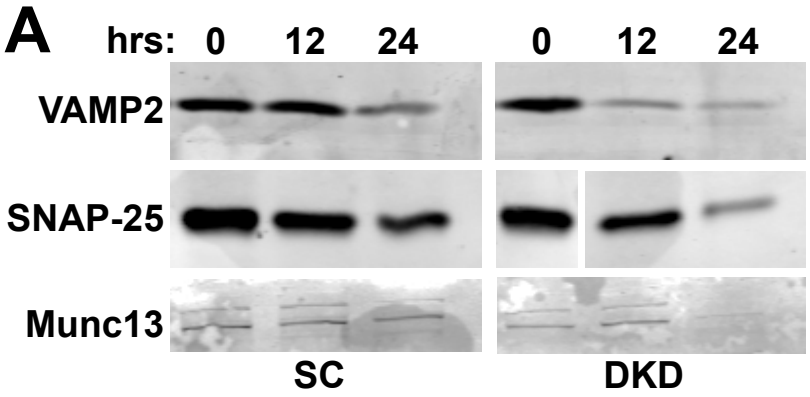


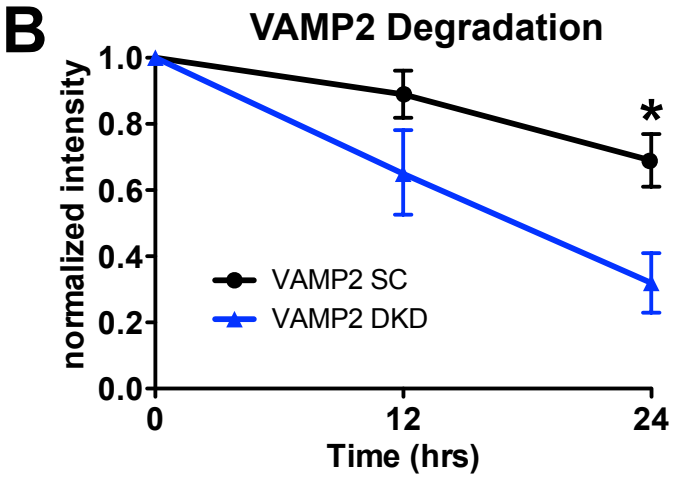


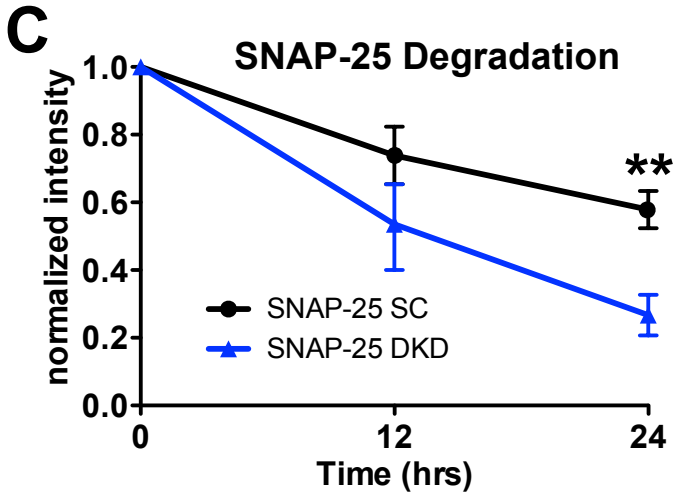
Waites et al. Fig. S1

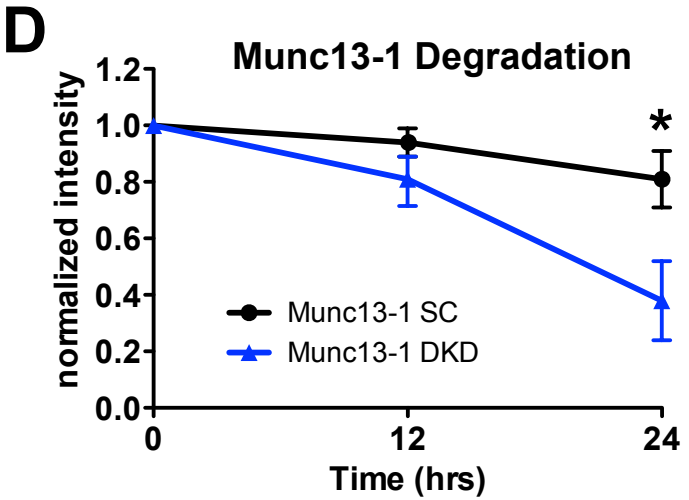


Waites et al. Fig. S2

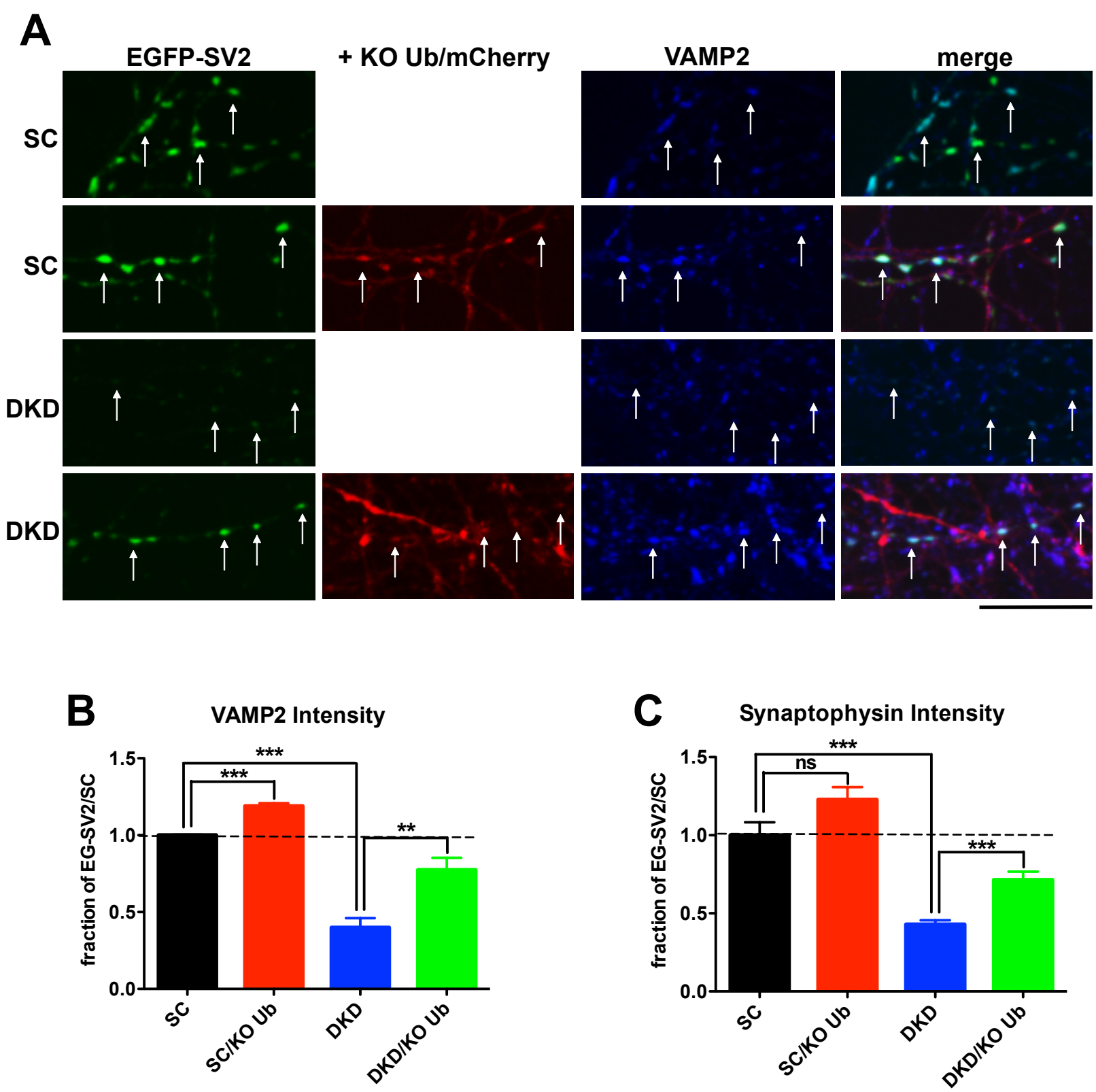




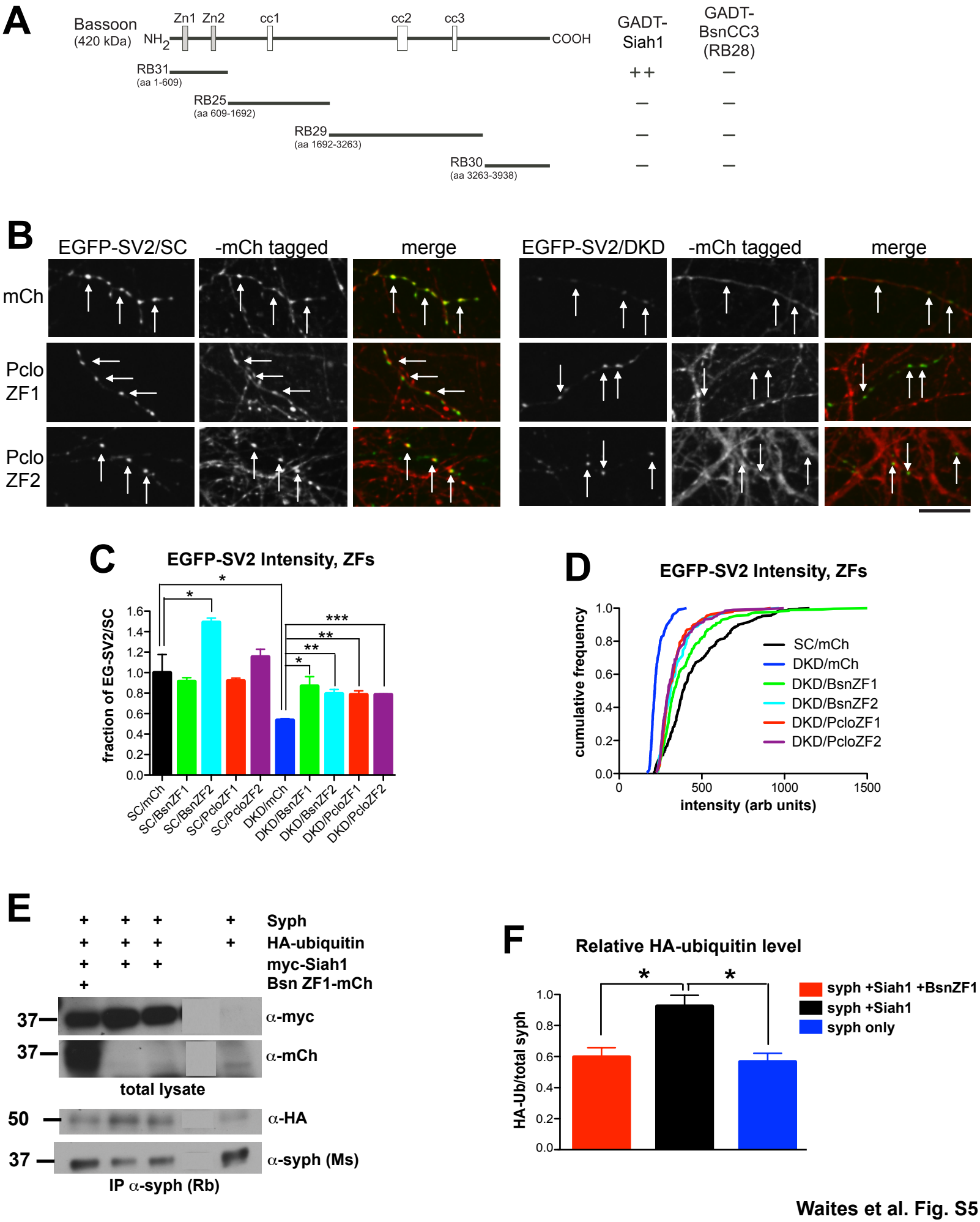




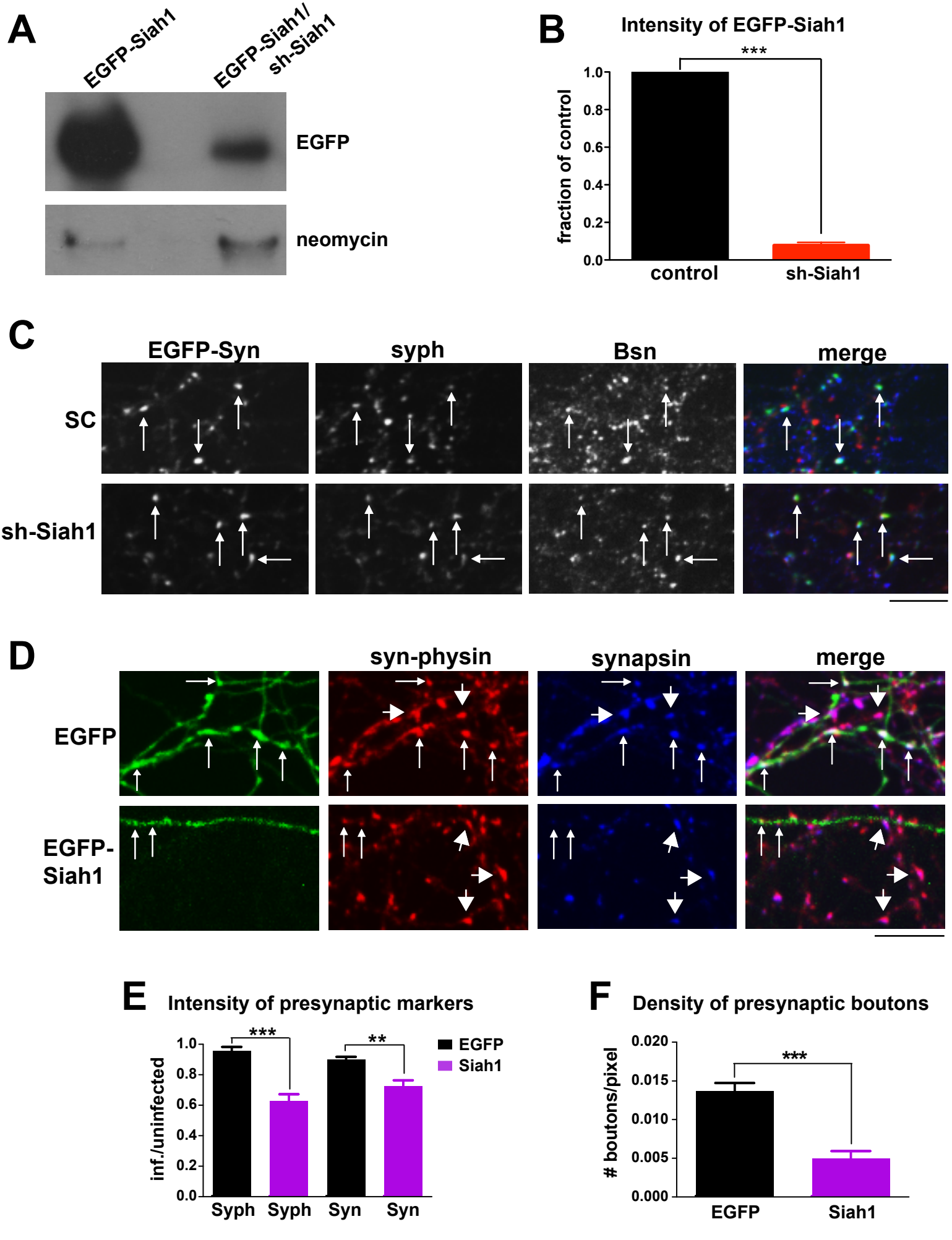
Waites et al. Fig. S3



Waites et al. Fig. S4



Waites et al. Fig. S5



Waites et al. Fig. S6